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Significance of Retinoblastoma Protein in Survival and Differentiation of Cerebellar Neurons

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1. Introduction

During development of the nervous system an excess number of neural progenitor cells are generated and approximately half of these cells are eliminated by programmed cell death (PCD) or apoptosis (Farinelli and Greene, 1996; Jacobson et al., 1997; Oppenheim, 1991; Raff, 1992; Raff et al., 1993). The apoptosis and elimination of the excess number of precursor cells enable the proper synaptic integration of the surviving cells and development of the central nervous system (CNS). Survival of the neurons in the CNS requires trophic support and electrical activity and upon withdrawal or depletion of these factors the neurons undergo apoptosis (Barde et al., 1987; Biffo et al., 1994; D'Mello et al., 1997; D'Mello et al., 1993; D'Mello et al., 2000; Galli et al., 1995; Levi-Montalcini, 1987; Miller and Johnson, 1996). Among the growth factors that support neuronal survival and differentiation are neurotrophic growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 and 4 (NT3 and NT4), insulin and insulin like growth factors (IGF), glial derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and ciliary neurotrophic factor (CNTF) (Ardelt et al., 1994; Barde, 1994; de Pablo et al., 1990a; de Pablo et al., 1990b; Ferrari et al., 1989; Ferrer et al., 1998; Hynes et al., 1994; Kalcheim et al., 1987; Knusel et al., 1990; Levi-Montalcini, 1987; Lindholm et al., 1993; Magal et al., 1993; Rabacchi et al., 1999; Rakowicz et al., 2002; Serrano et al., 1990; Tuttle et al., 1994; Zhang et al., 1997).

Many neurological diseases such as Alzheimer's, tauopathies, Parkinson's etc., show neuronal loss in specific areas of the brain (Burke, 1998; Cotman and Anderson, 1995; Cotman and Su, 1996; Forloni, 1993; Gorman et al., 1996; Hajimohamadreza and Treherne, 1997; Hartmann and Hirsch, 2001; Honig and Rosenberg, 2000; Jellinger, 2001; Johnson, 1994; Savitz and Rosenbaum, 1998; Yanagisawa, 2000; Yuan and Yankner, 2000). Although a number of signaling pathways have been implicated in the apoptosis observed in the brains it is difficult to determine whether inhibition of these pathways has any effect on neuronal survival *in vivo*. Therefore, in order to understand the *in vivo* mechanisms involved in neuronal apoptosis, researchers mainly use either transgenic mouse models or *in vitro* cultures of dissociated primary neurons or organotypic slice cultures from different brain regions from rodents. The mechanisms by which the different types of neurons undergo

apoptosis vary, and it depends on the type of insult as well as the type of neurons involved. For example, studies in sympathetic ganglia have shown that growth factor withdrawal and oxidative stress-induced apoptosis is associated with caspase activation and cyclin D1 expression (Freeman et al., 1994; Stefanis et al., 1998; Troy et al., 1997; Troy et al., 1996). Inhibitors of caspases such as z-DEVD-FMK and z-VAD-FMK protected these neurons from undergoing apoptosis thereby confirming that indeed caspase activation is involved in cell death. Similarly insults such as Aβ treatment, growth factor deprivation and treatment with DNA damaging agents induce apoptosis in cortical neurons, which is associated with activation of caspases as well as cell cycle regulatory proteins (Park et al., 1997a; Park et al., 1997b; Park et al., 1998a; Park et al., 1998b; Stefanis et al., 1999; Troy et al., 2000; Troy et al., 2001). Activation of cell cycle regulatory mechanisms have also been implicated in cerebellar granule neurons (CGNs) undergoing activity withdrawal-induced apoptosis (Konishi and Bonni, 2003b; Konishi et al., 2002; O'Hare et al., 2000; Padmanabhan et al., 1999).

It is now established that in neurons subjected to apoptotic insults, retinoblastoma protein (Rb) undergoes cdk-mediated phosphorylation which leads to its inactivation and dissociation from E2F (Boutillier et al., 1999; O'Hare et al., 2000; Padmanabhan et al., 1999; Park et al., 1997a; Park et al., 1997b; Park et al., 1998a; Sakai et al., 1999). Dissociated E2Fs induce transactivation of specific proapoptotic genes and apoptosis in different types of cells including neurons. In addition to transcriptional activation, derepression of proapoptotic genes is also implicated in neurons undergoing apoptosis. For example, studies have shown that the transcription factors B-Myb and C-myb are induced in cortical neurons subjected to growth factor withdrawal and DNA damage mediated apoptosis (Liu et al., 2004; Liu and Greene, 2001). It was found that the antisense RNA-mediated down regulation of B-myb and C-myb protected neurons from undergoing apoptosis and overexpression of these transcription factors was sufficient to induce apoptosis. This suggests that cell cycle activation in neurons induce dissociation of Rb/E2F complex leading to derepression and transactivation of proapoptotic genes.

This chapter mainly focuses on the mechanisms involved in neuronal apoptosis in cerebellum. Within the developing brain, cerebellar cortex has been extensively used for studying neuronal survival and apoptosis. The cerebellum plays a major role in movement, motor coordination, learning and cognitive function (Wechsler-Reya and Scott, 2001). It contains different types of neurons, of which Purkinje neuron is the most elaborate with a large cell body and vast dendritic tree. Due to the abundance of cerebellar granule neurons (CGNs) they have been used widely to study molecular mechanisms of neurodegeneration. The granule neurons differentiate into mature neurons when cultured in the presence of appropriate growth factors or when supported by depolarizing concentrations of KCl (D'Mello et al., 1997; D'Mello et al., 1993; Miller and Johnson, 1996). Growth factors that usually support survival of CGN are serum and IGF-1. Treatment of CGNs with high concentrations of KCl (25 to 30 mM) induces electrical activity and membrane depolarization, which allows Ca2+ entry through voltage sensitive calcium channels (Catterall, 2000; Konishi and Bonni, 2003b). Growth factor removal and KCl withdrawal induce neuronal apoptosis in CGNs, which involves different types of signaling pathways. This chapter mainly focuses on the involvement of cell cycle regulatory proteins in cerebellar granule and Purkinje neuron apoptosis and highlights the importance of functional retinoblastoma protein in survival and maintenance of differentiated neurons.

2. Retinoblastoma protein

Retinoblastoma protein (Rb) is a negative regulator of cell cycle progression and is known as the master regulator of cell cycle, differentiation, senescence and apoptosis (Chen et al., 1995; Dasgupta et al., 2006; Herwig and Strauss, 1997; Knudsen et al., 2000; Lee et al., 1995; Riley et al., 1994; Wang, 1997; Wang et al., 1994; Weinberg, 1989a, b, 1990, 1991, 1995). It belongs to a family of proteins known as pocket proteins, which include p107 and p130. These proteins bind to the early transcription factors (E2Fs) and control the G1/S transition of cells. Rb associates with several members of the E2F family and inhibits transactivation of E2F-responsive genes. Among the E2F family members (E2Fs 1 through 8) E2Fs 1, 2, and 3 are transcriptional activators and have been shown to associate with Rb. The binding of Rb to E2F depends on the phosphorylation state of Rb (Angus et al., 2002; Chellappan et al., 1991; Hiebert et al., 1992; Nevins et al., 1991). A cell, upon receipt of growth factor or different proliferative signals, induce expression of cyclin D in the early G1 phase and cyclin E in the later G1 phase. These cyclins associate with the respective cyclin-dependent kinases (cdk). Cdk4 and cdk6 associate with cyclin D1 while cdk2 interacts with cyclin E. The cyclin-cdk complex induces phosphorylation of Rb leading to its inactivation and dissociation from E2F which results in G1/S checkpoint release and E2F-dependent transcriptional activation (Figure 1). Thus, functional retinoblastoma protein plays a major role in control of cell division and loss of its function by mutation, phosphorylation or degradation leads to uncontrolled cell division and tumorigenesis. After G1/S checkpoint release, further progression of cells through S and G2/M phases are brought about by cyclin A/cdk2 (S-phase) and cyclin B/cdc2 (cdk1) complexes, respectively. In addition to the cdks, the *in vivo* inhibitors of cdks (CKIs) such as p16, p21, p27 and p57 also play a role in cell cycle control (Besson et al., 2008). Thus, cell cycle is tightly regulated by the combined efforts of cyclins, cdks, cyclin-dependent kinase inhibitors (CKIs) and Rb.

In addition to its anti-proliferative role, retinoblastoma protein can also function as an anti-apoptotic factor. Rb exerts its growth-inhibitory effects mainly by binding and inhibiting transactivation of E2F family of transcription factors (Chellappan et al., 1991; Dasgupta et al., 2004; Nevins et al., 1991; Stevaux and Dyson, 2002). Among these transcription factors, E2F1 has been implicated in not only S-phase entry but also apoptosis induction through the p53 and p73 pathways (Irwin et al., 2000; Lissy et al., 2000; Zaika et al., 2001). Overexpression of Rb inhibits E2F1-mediated apoptosis. It has been suggested that the increased apoptosis observed in Rb null mice is brought about mainly by increased E2F1 activity. Studies by Chellappan and colleagues have shown that in addition to the cyclins and cdks, non-cyclin dependent kinases can also phosphorylate and inactivate Rb (Dasgupta et al., 2004; Nath et al., 2003; Wang et al., 1999a; Wang et al., 1999b). For example, during mitogenic signaling, Raf1 directly interacts with and phosphorylates Rb. Analysis of Rb-associated mechanisms in cells undergoing apoptosis showed an interaction of Rb with kinases such as p38 MAP kinase and apoptosis signal regulating kinase 1 (ASK1) (Dasgupta et al., 2004). This suggests that in addition to the cdks Rb can be phosphorylated and inactivated by non-cyclin-dependent kinases as well.

Several viral oncoproteins such as the SV-40 large T-antigen (T-Ag), E1A of the adenovirus and E7 of the human papilloma virus type 16 have been shown to bind Rb through an LXCXE motif. Interaction of Rb with viral oncoproteins lead to dissociation of Rb from E2F and induction of E2F-dependent gene expression (Chellappan et al., 1992; Chellappan et al.,

1991; Hiebert et al., 1992; Nevins et al., 1991). Loss of Rb function leads to an increase in p53 activity via an E2F-dependent induction of ARF family of proteins. ARF induces degradation of MDM2 and stabilization of p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Tao and Levine, 1999; Weber et al., 2000; Zhang et al., 1998). This is one of the mechanisms by which Rb induces p53-dependent apoptosis in non-transformed cells. Similarly, human cytomegalovirus (HCMV) has been shown to inhibit the Rb-E2F association. The HCMV-mediated Rb inactivation was not inhibited by the cdk inhibitors roscovitine, olomoucine or flavopiridol. *In vitro* and *in vivo* studies have shown that HCMV-mediated Rb phosphorylation is brought about by UL97, an HCMV protein kinase, and inhibition or inactivation of this kinase can prevent Rb phosphorylation (Hume et al., 2008).

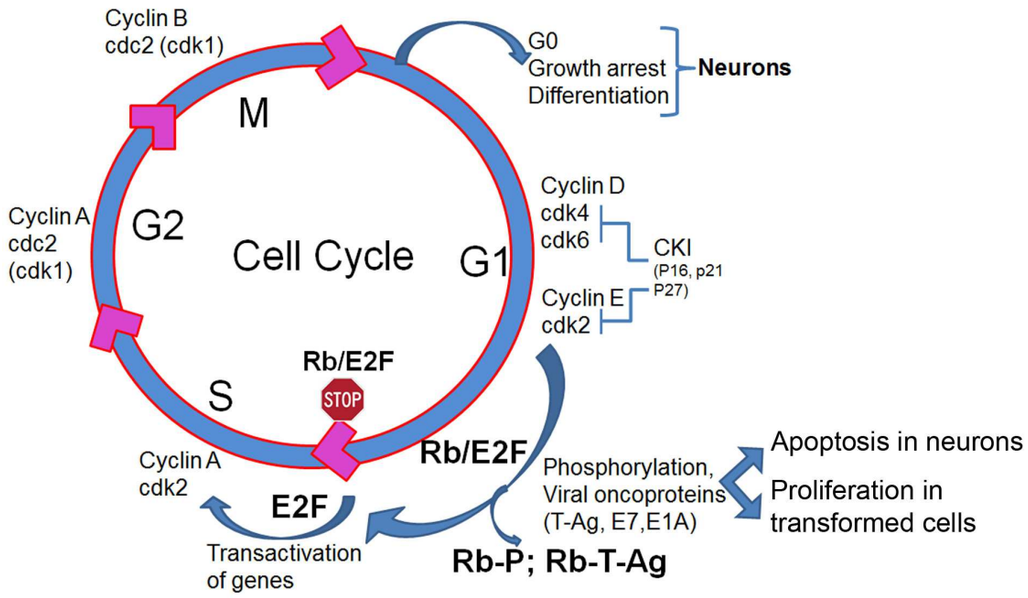

Fig. 1. Retinoblastoma (Rb) protein and cell cycle: In resting cells Rb associates with E2F and prevents (STOP) transition of cells through the G1→S checkpoint. Upon receipt of growth factors or proliferative signals, cyclins and cdks are activated leading to phosphorylation of Rb (Rb-P) and release of Rb from E2F. In the early G1 phase cyclin D associates with cdk4 or cdk6 and in the late G1 phase cyclin E associates with cdk2 to induce phosphorylation and inactivation of Rb. The activities of the cdks are regulated by cyclin-dependent kinase inhibitors (CKI). These CKIs, such as p16, p21 and p27, inhibit cdks from phosphorylating and inactivating Rb. In addition to phosphorylation, binding of viral oncoproteins such as SV-40 large T-antigen (T-Ag), E1A of adenovirus and E7 of papilloma virus can also inactivate Rb. Binding of these oncoproteins to Rb (example, Rb-T-Ag) releases Rb from E2F resulting in transactivation of E2F-dependent genes needed for cell proliferation. Once the cells pass through the G1/S checkpoint, further progression through cell cycle is made possible by cyclin A/cdk2 in S phase and Cylin B/cdc2 (cdk1) in the G2-M phase. Fully developed mature neurons are differentiated cells and are retained in the G0 phase of the cell cycle. Unlike proliferating cells which undergo transformation and uncontrolled proliferation upon Rb inactivation, neurons undergo neurodegeneration and apoptosis demonstrating the importance of Rb in maintenance of healthy differentiated neurons.

3. Retinoblastoma protein and neuronal survival

In addition to its function in control of cell cycle, Rb has been shown to be important in development and survival of neurons (Athanasiou et al., 1998; Feddersen et al., 1995; Feddersen et al., 1997; Hoglinger et al., 2007; Padmanabhan et al., 2007). Although it is known that precursor cells can divide and the newly formed daughter cells can migrate and differentiate into mature neurons, the ability of mature neurons to divide is debatable. Mature neurons are usually maintained in the G_0 or the resting phase of the cell cycle and respond to cell cycle activation by undergoing apoptosis rather than transformation. This is further supported by the fact that there are rarely any cases of tumors that originate from mature neurons. Several lines of evidence indicate that neuronal development and survival requires the presence of functional Rb in the nervous system. Mice lacking Rb show defects in neurogenesis and die embryonically at day 16 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). This shows that Rb is important for proper exit of immature precursor cells from cell cycle and generation of mature differentiated neurons, and in the absence of Rb they attempt to reenter cell cycle but undergo neurodegeneration and apoptosis.

4. Degeneration of cerebellar neurons

4.1 Cerebellar granule neurons

Cerebellar granule neurons (CGN) when cultured in the presence of serum and depolarizing concentrations of KCl (25 mM) acquire characteristics of fully differentiated mature neurons similar to those present *in vivo* (Galli et al., 1995). Upon lowering the concentration of KCl to 5 mM these neurons undergo apoptosis. Apoptosis in CGN is prevented by treatment with IGF-1, cyclic AMP, forskolin and inhibitors of transcription and translation (D'Mello et al., 1997; D'Mello et al., 1993; Miller and Johnson, 1996; Padmanabhan et al., 1999). The protection of cells by transcriptional inhibitors point to the fact that under apoptotic conditions the transcriptional machinery is activated. Since cell cycle dependent mechanisms are under transcriptional regulation it was hypothesized that neuronal apoptosis may be associated with deregulation of cell cycle.

Although neuronal apoptosis has been shown to be associated with activation of caspases, no significant protection was observed by caspase inhibitors upon activity withdrawal-induced apoptosis in CGNs. This led to the analysis of the role of cell cycle regulatory proteins in this apoptosis paradigm (Padmanabhan et al., 2007). Neurons were deprived of KCl in the presence of inhibitors of cdks and their survival was examined at different time points. It was found that the cdk inhibitors could provide significant protection of rat CGN from KCl deprivation induced apoptosis even when the caspase acitivity was high (Padmanabhan et al., 1999). The discrepancy in the results from different laboratories could be explained by the age of the culture, origin of the cells (mouse vs rats), or the culture conditions. It is possible that lowering the KCl concentrations to 5 mM without withdrawal of serum may use a different mechanism to induce apoptosis compared to that induced by withdrawal of both serum and KCl. It is known that KCl withdrawal is associated with induction in caspase activity and treatment of the neurons with a general caspase inhibitor can significantly prevent the increase in activity. This suggests that irrespective of the status of caspases in the cells, transcriptional activation clearly leads to KCl withdrawal-induced cell death in CGN. Analysis of cyclin D1 and cyclin E showed that these cylcins are normally

present in the CGNs. Upon KCl withdrawal, the cells begin to undergo a time-dependent apoptosis and the expression of these cyclins is induced. Immunostaining analysis revealed that neurons undergoing apoptosis accumulate cyclin D1 in their nucleus which is indicative of its role in activation of cell cycle (Padmanabhan et al., 1999). This result is different from what has been observed in sympathetic neurons wherein the expression of cyclin D1 is non-detectable under non-apoptotic conditions and is detectable only upon growth factor deprivation-induced apoptosis. This shows that the gene expression patterns vary between neurons from different regions and therefore may respond differently to diverse external cues.

Immunoprecipitation and kinase activity assays showed that apoptosis in CGN is associated with an induction in cyclin D1 and cyclin E-associated kinase activities (Padmanabhan et al., 1999). *In vivo* inhibitors of cdks such as p16, p21, p27 and p57 (CKIs) regulate the activities of cdks. It was found that the neurons undergoing apoptosis show a time-dependent decrease in p27 levels (Martin-Romero et al., 2000; Padmanabhan et al., 1999). Decreased p27 levels in turn, enhance the cdk activity further and enable cdks to phosphorylate and inactivate Rb resulting in dissociation of Rb from E2F. This leads to enhanced E2F-dependent transactivation or derepression of proapoptotic genes. Analysis of the neurons showed that Rb phosphorylation and degradation are enhanced in cells undergoing apoptosis (Padmanabhan et al., 1999). Since Rb is the major regulator of G1→S phase transition, this observation indicates that the neurons are forced to exit the G0 phase and re-enter the cell cycle. Although the neurons exit the resting phase of the cell cycle they do not undergo cell division or transformation but instead undergo apoptosis.

Treatment of the neurons with inhibitors of cdks protected CGNs from KCl withdrawal-induced apoptosis (Padmanabhan et al., 1999). Flavopiridol, a flavonoid that is specific to cdk 1, 2 and 4, at a concentration of 1 μM and olomoucine and roscovitine, purine derivatives with specificity towards cdk 1, 2 and 5, at concentrations of 200 μM and 50 μM, respectively, protected CGNs from KCl withdrawal-induced apoptosis. This was associated with inhibition of the cdk activities, inhibition of translocation of cyclin D1, prevention of degradation of p27, and inhibition of Rb phosphorylation and degradation. Cdk4 and cyclin D as well as cdk2 and cyclin E activities are essential for Rb phosphorylation and transition of cells through G1/S checkpoint. Thus, this study suggests that upon activity withdrawal CGNs attempt to enter the cell cycle but due to the lack of an active cell cycle program this attempt is aborted and the cells take the alternative approach and undergo apoptosis. Studies in mouse neurons have also shown induction of cyclin D1 and associated kinase activity upon KCl withdrawal-induced apoptosis. This was associated with Rb hyperphosphorylation and degradation which could be inhibited by treatment with caspase inhibitors (Boutillier et al., 1999; Boutillier et al., 2000). Expression of a caspase cleavage mutant of Rb protected the cells from undergoing apoptosis thereby suggesting that physiological levels of functional Rb is necessary for survival of CGNs. This again suggests that upon lowering KCl concentrations, the neurons attempt to re-enter cell cycle but due to the absence of an active cell division cycle, they undergo apoptosis.

Since E2F1 has been implicated in apoptosis it is rational to think that E2F1 overexpression may lead to cell death. Analysis of CGNs undergoing apoptosis showed that E2F1 mRNA and protein levels were induced upon KCl withdrawal (O'Hare et al., 2000). This study also showed that adenovirus mediated overexpression of E2F1 in CGNs can induce apoptosis.

This apoptosis was p53-independent as overexpression of E2F1 in neurons from both p53+/+ and p53-/- mice showed similar levels of apoptosis. The E2F1-mediated apoptosis was found to be Bax-dependent and was associated with increased caspase 3-like activity. Studies conducted on cells from E2F1 deficient mice showed significantly higher number of surviving neurons upon withdrawal of KCl confirming that E2F1 expression is associated with enhanced apoptosis in postmitotic neurons.

Studies conducted on neurons treated with kainic acid have shown that it is associated with a transient increase in Rb phosphorylation suggesting a role for aberrant cell cycle activation (Giardina et al., 1998). When the effect of kainate treatment was compared in neurons from E2F1 deficient and E2F1 WT neurons, it was found that the neurons from E2F1 -/- mice were more resistant to KA-induced apoptosis. Thus, this study shows that excitotoxicity-induced apoptosis in neurons is also mediated through cell cycle activation, inactivation of Rb and E2F1-dependent transactivation of genes.

The above studies clearly show that activation of the components of G1 phase of cell cycle is associated with activity winthdrawal-induced apoptosis in CGNs . None of them show any evidence for entry of cells in to the S phase or expression of any of the late phase markers of cell cycle upon induction of apoptosis. It was hypothesized that the cells undergoing apoptosis acquire morphology similar to those undergoing mitosis and therefore mechanisms similar to that seen in mitotic phase of cell cycle may be activated in the apoptotic process (King and Cidlowski, 1995). This hypothesis was supported by the studies in fibroblasts where cdc2 has been shown to induce apoptosis (Yu et al., 1998). In order to determine whether cdc2 expression is associated with apoptosis in neurons, Konishi and colleagues examined CGNs undergoing activity winthdrawal-induced apoptosis (Konishi et al., 2002). Their studies showed that activity withdrawal-induced, but not growth factor withdrawal-induced, cell death in CGN is associated with induction in the G2/M kinase cdc2 (cdk1) and cdc2-mediated BAD phosphorylation. Cdc2 associates with cyclin B in the G2/M phase of cell cycle and regulates the onset of M-phase. Experiments in CGN deprived of depolarizing concentrations of KCl showed that cdc2 kinase enhanced the phosphorylation of the proapoptotic protein BAD at Ser128 residue upon apoptosis. Under normal conditions growth factor-induced phosphorylation of BAD at Ser136 leads to its sequestration by 14-3-3 proteins thus preventing it from inducing apoptosis. Under apoptotic conditions, the additional phosphorylation of BAD at Ser128 by the cdc2 kinase prevents it from getting sequestered by 14-3-3 resulting in BAD-induced apoptosis in neurons.

Cdc2 is an E2F responsive gene and induction in E2F transcriptional activity may therefore upregulate the expression of cdc2. Chromatin immunoprecipitation (ChIP) assays using E2F1 antibody and analysis of the promoter of cdc2 kinase has shown that E2F forms a complex with the promoter endogenously thereby suggesting that E2F1 can induce transactivation of cdc2 (Konishi and Bonni, 2003a). It was also shown that the expression of a dominant negative E2F1 inhibits and WT E2F1 induces cdc2 expression and apoptosis of CGN. These observations clearly show that activity withdrawal-induced apoptosis in neurons is associated with cell cycle activation, Rb phosphorylation and inactivation, and G1→S transition in CGN. It appears that the neurons may even enter the G2/M phase of cell cycle before succumbing to apoptosis. Further, the phosphorylation of BAD by cdc2 kinase reveals how cell cycle mechanisms link to the cell death machinery to bring about the apoptosis in neurons.

The cdks mentioned above are mainly exerting their effects by association with specific cyclins. For example cyclin D associates with cdks 4 and 6, cyclin E with cdk2, and cyclin A and cyclin B with cdc2. One cdk that does not depend on a cyclin to exert its activity and is mainly active in the nervous system is the cyclin dependent kinase 5 (cdk5) (Tsai et al., 1994). The regulatory subunits that activate this cdk are the p35 and p39 which are found in the neuronal tissue. P35-cdk5 complex is expressed at high levels in the adult brain and is involved in neuronal migration and axonal growth (Nikolic et al., 1996; Ohshima et al., 1999). P35 is proteolytically cleaved by calcium-dependent proteases to generate p25, which is more stable and active. The p25-cdk5 complex is hyperactive and has been shown to induce neurotoxicity (Lee et al., 2000; Patrick et al., 1999). Studies conducted using embryonic mouse brain extracts as well as bacterially expressed Rb and cdk5/p25 have shown that p25 can directly bind to Rb and induce its phosphorylation (Lee et al., 1997). In addition, studies in SY5Y cells overexpressing inducible p25 showed that it enhances phosphorylation of Rb which is blocked by roscovitine, a kinase inhibitor that inhibits cdks 1, 2 and 5 but not the cyclin D kinases cdks 4 or 6 (Hamdane et al., 2005). These findings that cdk5 can phosphorylate and inactivate Rb suggest that even in the absence of alterations in cyclins and the associated cdks, this neuronal cdk5 may induce transcriptional activation and neurodegeneration by causing inactivation of Rb.

4.2 Purkinje neurons

Extensive studies by Herrup and colleagues have shown that the different types of neurons in the cerebellum depend on each other, especially on the Purkinje neurons for trophic support and survival (Wetts and Herrup, 1982, 1983). This is termed as 'developmental dependency'. A considerably high number of cerebellar granule neuron precursor cells are generated during brain development. Studies have shown that numerical matching of the granule cells to Purkinje cells is important for normal cerebellar development and the excess number of cells that do not reach the target or that do not connect with the Purkinje cells are eliminated by apoptosis (Herrup and Sunter, 1987; Vogel et al., 1989). In the case of Purkinje neurons only a limited number of immature cells are generated which develop into the mature Purkinje cells in a cell autonomous way. Studies done in *Staggerer* and *Lurcher* mutant mice have shown that loss of Purkinje neurons is associated with loss of CGNs and inferior olive neurons (Herrup and Mullen, 1979; Rabacchi et al., 1992; Sonmez and Herrup, 1984; Vogel et al., 1991). These mutant mice show defects in the development of Purkinje neurons. In the *Staggerer* mice the Purkinje neurons never develop fully resulting in deficiency in the targets required for CGN to establish contacts. This leads to loss of neurons and these mutant mice show 100% loss of CGNs. On the other hand in the *Lurcher* mice a small percent (10%) of the CGNs survive even when 100% of the Purkinje neurons die between postnatal day 9 and 30. Analysis of CGNs and inferior olive neurons in these mice revealed that the death is associated with enhanced expression of cyclin D1, PCNA and increased DNA synthesis, as evident by BrdU incorporation (Herrup and Busser, 1995). This suggests that lack of trophic factor support induces cell cycle reentry and cell death in these neurons *in vivo*. PCNA is an S phase specific marker and this along with the induction of DNA synthesis in neurons undergoing apoptosis suggests that their death may be associated with inactivation of Rb and induction of E2F-dependent transactivation of genes. The degenerating Purkinje cells do not show any incorporation of BrdU which is indicative of a different mechanism involved in the death of these neurons in these mutant mice.

The absence of cell cycle activation in degenerating Purkinje neurons in *Staggerer* and *Lurcher* mutant mice does not mean that these cells do not re-enter cell cycle. Studies conducted in mice overexpressing the viral oncoprotein SV40 large T-antigen (T-Ag) have shown that Purkinje cell specific overexpression of T-Ag is associated with cell cycle activation and neurodegeneration (Feddersen et al., 1995; Feddersen et al., 1997). T-Ag overexpressing mice show DNA synthesis and nuclear fragmentation indicative of programmed cell death. Further, analysis of the Purkinje neurons using mutated T-Ag showed that overexpression of the Rb binding domain of this oncoprotein is sufficient to induce neurodegeneration of Purkinje neurons indicating that functional Rb is essential for the survival of these neurons.

Depending on the levels of T-Ag expression in the Purkinje cells, the mice showed variation in development of neurodegeneration and ataxia (Feddersen et al., 1997). Mice that express greater than 30 copies of the T-Ag transgene showed ataxia at 2 weeks of age. This was associated with immature Purkinje cell death and defects in cerebellar development. This suggests that normal development of Purkinje cells is essential for the proliferation, differentiation and migration of cerebellar granule cells from external to internal granule layer. Mouse with 10 copies of transgene showed ataxia at 10 weeks and those with 2 copies at 15 weeks. This study clearly shows the importance of functional Rb in terminal differentiation and protection of neurons and explains the reason for detection of high levels of Rb in the adult brain (Bernards et al., 1989; Okano et al., 1993). It has been shown that the final mitosis in Purkinje cells occurs at day 13. In mice expressing T-Ag the loss of Purkinje neurons due to cell cycle activation occurred at day 14 (2 weeks) suggesting that developing Purkinje neurons are incapable of initiating cell division.

Since E2F1 overexpression has been implicated in apoptosis, Feddersen and colleagues examined whether the levels of E2F1 is enhanced in degenerating Purkinje cells (Athanasiou et al., 1998). They found that both E2F1 and the E2F-responsive cdc2 gene were induced in the same neurons indicating the E2F-dependent transactivation of genes upon apoptosis induction. This prompted them to look at the effect of overexpression of E2F1 in Purkinje neurons. Their studies showed that overexpression of E2F1 by itself did not have any profound effect on Purkinje cell morphology or survival. But, the E2F1 overexpressing Purkinje cells showed accelerated neurodegeneration upon T-Ag overexpression suggesting that either a posttranslational modification or an association of E2F with other regulators such as dimerization partner 1 or 2 (DP1 and DP2) is necessary for induction of transactivation of genes (Athanasiou et al., 1998).

Normally, T-Ag overexpression leads to tumorigenesis in mouse tissues, including neurons of retina and CNS (al-Ubaidi et al., 1992a; al-Ubaidi et al., 1992b; Hammang et al., 1990), but in the case of Purkinje neurons it was associated with neurodegeneration, apoptois and ataxia (Feddersen et al., 1995). This suggests that T-Ag induces differential effects in different types of cells in a context specific manner, and indicates the significance of functional Rb in normal development and differentiation of the Purkinje neurons. In this context, studies in photoreceptor cells of the retina showed that if oncogene overexpression is induced prior to cessation of mitosis, it leads to tumorigenesis whereas overexpression in postmitotic cells leads to degeneration and apoptosis (al-Ubaidi et al., 1992a; al-Ubaidi et al., 1992b; Feddersen et al., 1995; Howes et al., 1994).

In vitro studies further confirmed the significance of functional Rb in survival and normal development and protection of Purkinje neurons (Padmanabhan et al., 2007). Examination of organotypic slice cultures of cerebellum taken from Sprague Dawley rats at postnatal day 4 (P4) and 9 (P9) showed a time-dependent decrease in survival of Purkinje neurons (Padmanabhan et al., 2007). It has been shown that the Purkinje neurons in slice cultures prepared from postnatal day P1 through P7 die by apoptosis (Dusart et al., 1997; Ghoumari et al., 2000). Treatment of the cultures with pharmacological inhibitors of cdks such as roscovitine, olomoucine and flavopiridol protected the neurons from undergoing apoptosis, with roscovitine showing the maximum effect (Figure 2).

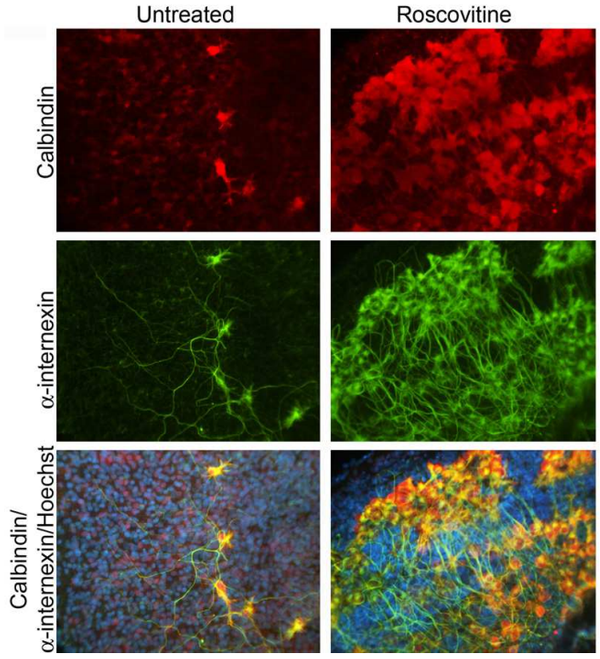

Fig. 2. Protection of Purkinje neurons by roscovitine in organotypic slice cultures: Cerebellar slice cultures from P4 rats were treated with or without 50 μM roscovitine for 1 week. Sections were fixed and stained using polyclonal calbindin (top row) and monoclonal α-internexin (middle row) antibodies. Alexa 594 fluorophore was used for detection of calbindin (red) and Alexa 488 for α-internexin (green). Hoechst was used to detect the nuclei which allowed us to view the integrity of the section. Bottom row show the composite image showing all the three staining. Sections were analyzed using a Nikon Eclipse E1000 fluorescent microscope and using Genus 2.81 software. Roscovitine protected the cell body of the neruons but not the dendrites.

Although the cdk inhibitor-treated slices showed significantly higher number of calbindin positive Purkinje neurons (Figure 2), the morphology of the cells was quite different. The elaborate dendritic arborization of the Purkinje neurons was not protected by the inhibitors; the dendritic tree showed a stunted appearance and the axons appeared to be shorter. The Purkinje neurons in the untreated sections, that survived the axotomy-induced apoptosis, showed normal dendritic arborization. This may suggest that the mechanism involved in the maintenance or protection of cell body is different from that involved in the protection of neurites. Another possibility is that the supporting cells

required for normal development of Purkinje neurons are not protected by the cdk inhibitor treatment and therefore the morphology of the neurons are not maintained. Granule cells have been shown to provide the trophical support and electrical activity required for the normal development of Purkinje neurons, and Bergmann glia is necessary for the directed growth and polarity of the Purkinje dendrites. Both Purkinje cells and granule cells have been shown to express BDNF and knockout mice for BDNF show stunted growth of Purkinje neurons and loss of granule neurons (Schwartz et al., 1997). In addition, growth factors such as GDNF, NGF, NT-3, CNTF, and IGF-1 have been implicated in survival of these neurons (Segal et al., 1997). Although we tried to rescue the morphology and cell death by providing trophic support, NT3 and BDNF, we did not observe any significant protection suggesting the involvement of a more complex mechanism in the maintenance and protection of Purkinje neurons.

Analysis of the sections with the α-internexin antibody, which stains the parallel fibers of CGNs showed loss of these neurons in the slices. As discussed earlier survival of these neurons depends on the development and support from the Purkinje neurons. The fact that the cdk inhibitors were unable to protect the dendrites and axons of the Purkinje neurons suggests that this may have a profound impact on the survival of CGNs and inferior olive neurons. Normally Purkinje neurons in slices taken from rats between postnatal days P1 and P5 die within 1 week after culturing (Dusart et al., 1997). Studies with the inhibitors showed that cerebellar sections taken from P4 rats show significant protection of Purkinje neurons after 1 week in culture (Padmanabhan et al., 2007). These results clearly indicate that the Purkinje neurons in the explants undergo apoptosis through a cell cycle dependent mechanism.

Since Rb has been implicated in the survival and maintenance of differentiated state of neurons, experiments were done to determine whether Rb can protect the Purkine neurons in the organotypic slice cultures. Overexpression of a WT and phosphorylation site mutant of Rb in cerebellar sections was achieved by adenovirus-mediated method (Padmanabhan et al., 2007). Rb overexpression showed significant protection of Purkinje neurons in the slice cultures. In addition, the neurons retained their normal dendritic arbors and axons further establishing the importance of functional Rb in protection and development of normal Purkinje neurons.

5. Relevance to diseases

5.1 Cerebellar tumors

Rb plays an essential role in cell proliferation, differentiation and migration of granule cell precursors in the cerebellum. Lack of Rb during cerebellar development results in prolonged proliferation, delayed differentiation, and altered migration of precursor cells. Improper differentiation of cerebellar neurons can initiate tumors of the cerebellum (Fults, 2005). For example, one of the most common malignant tumors of the childhood is the medulloblastomas. This is a tumor of the cerebellum and it originates from transformed granule cell precursors. Sonic Hedgehog (Shh), Wingless (Wnt) and Notch signaling pathways have been implicated in proliferation, differentiation and migration of granule cells (Katoh and Katoh, 2009; Oliver et al., 2003; Wechsler-Reya and Scott, 1999). Activating mutations in Shh have been implicated in granule precursor cell proliferation and

development of basal cell carcinoma and medulloblastoma. Shh-dependent cell proliferation in granule neuron precursor cells has been shown to be associated with expression of cyclins D1, D2 and E thereby promoting the cyclin-Rb pathway (Kenney and Rowitch, 2000). Granule cells in the cerebellum are generated in the external layer of cerebellum and migrate to the internal layer upon maturation. Studies in mouse models of medulloblastoma showed that granule cells in external granule layer (EGL) are involved in the development of medulloblastoma. These granule cells are immature and mitotic and when they become postmitotic, they migrate to the internal granule layer (IGL). Overexpression of Rb induced apoptosis in the cells derived from medulloblastomas implying that functional Rb is essential for the proper development, differentiation and migration of granule precursor cells. In the absence of Rb the precursor cells may continue to proliferate in the EGL layer and develop into malignant tumors.

Similar to Shh signaling, Wnt signaling has also been implicated in the development of medulloblastoma. Wnt functions through its association with the receptor Frizzled and by modulating the levels of β-catenin in the cells (Morin, 1999). When Wnt signaling is absent GSK3β phosphorylates β-catenin leading to its ubiquitination and degradation. Activation of Wnt signaling inactivates GSK3β leading to the stabilization of β-catenin. β-catenin translocates into the nucleus and transactivates LCT/TCF family of transcription factors inducing expression of genes such as c-Myc and cyclin D1 leading to aberrant cell cycle activation and cell proliferation. This also suggests that altered Wnt signaling in cerebellum can lead to activation of cyclin D-Rb axis and induction in transactivation of genes.

5.2 Ataxias

Another pathological condition that originates from defects in cerebellum is ataxia. Cerebellum plays a major role in motor coordination and movements as well as cognitive functions and damages to the cerebellum leads to loss of these functions. Studies conducted in transgenic mice with Purkinje specific expression of T-Ag showed that the oncogene expression is associated with degeneration of Purkinje neurons and developmental defects in the cerebellum (Feddersen et al., 1997). These mice developed ataxia that is characteristic of cerebellar dysfunction. T-Ag, as discussed before, associates with Rb, inhibits its binding to E2F1, and induces transactivation of genes. This suggests that cell cycle activation may play a critical role in different types of ataxia where cerebellar degeneration is a major contributor. These include Friedreich's ataxia, Ataxia Telangiectasia, congenital cerebellar ataxia etc. Loss of neuronal cell cycle control has been implicated in Ataxia-Telangiectasia, where Ataxia Telangiectasia gene is mutated (ATM), which is a neurological condition where progressive degeneration of neurons leads to major neuropathological disability (Kuljis et al., 1997; Yang and Herrup, 2005). This disease is associated with severe atrophy of the cerebellar cortical layers with extensive Purkinje and granule cell loss, dentate olivary nuclei atrophy, neuronal loss in the substantia nigra and oculomotor nuclei, spinal cord atrophy, and degenerative changes in spinal motor neurons (Crawford, 1998). The molecular mechanisms involved in the occurrence of this disease are unclear. Studies in ATM -/- mouse models have shown that vulnerable neurons in the cerebellum show ectopic expression of cell cycle proteins, which may indicate involvement of Rb and E2F (Yang and Herrup, 2005). Further studies are necessary to understand the exact molecular mechanisms involved in this and other ataxias.

6. Conclusion

In summary, *in vivo* studies conducted in mouse cerebellum from transgenic mice expressing viral oncoproteins (T-Ag), and mouse expressing neurological mutations such as *Staggerer* and *Lurcher* show that defects in development of Purkinje neurons and cerebellar granule neurons lead to abnormal development of the cerebellum and the development of ataxia. Studies with the T-Ag clearly show that the degeneration in Purkinje neurons leads to migratory defects in granule neurons and developmental defects in cerebellum. This is mainly caused by the loss of functional Rb leading to untimely cell cycle reentry of postmitotic neurons. This attempt by the postmitotic neurons to re-enter the cell cycle leads to catastrophic effects, and cells undergo apoptosis, suggesting that differentiated neurons need to be kept under tight control from re-entering the cell cycle. Once the cell cycle paradigm is activated, there is no return to the healthy state and the cells activate the apoptotic machinery to eliminate themselves. Prevention of this re-entry and maintenance of the neurons in the postmitotic state is critical for normal functioning of the brain. The *in vitro* studies in dissociated cerebellar granule neurons and organotypic slice cultures of cerebellum clearly show that both granule neurons and Purkinje neurons undergo cell cycle-mediated neurodegeneration and apoptosis (schematic, Figure 3). Although cdk inhibitors protected the neurons from undergoing apoptosis, the morphology of Purkinje neurons in the inhibitor treated cells looked abnormal. When protected by overexpression of Rb the Purkinje neurons showed very close to or normal dendritic and axonal development. This clearly suggests that the anti-apoptotic and anti-proliferative properties of Rb are essential

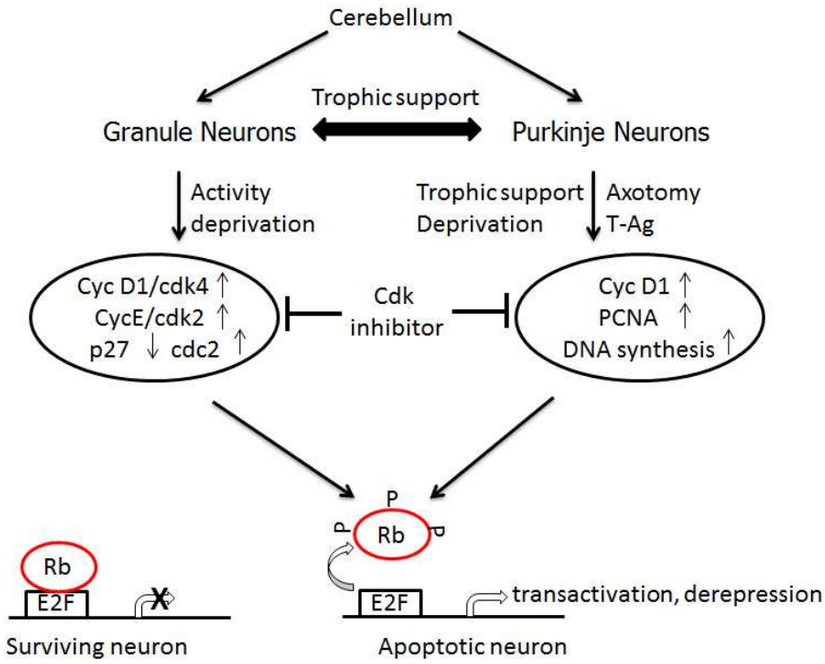

Fig. 3. Schematic showing the Rb-E2F pathway in neuronal apoptosis: Deprivation of activity or trophic facor support induces cyclins and cdks which phosphorylate Rb, release Rb from E2F and induce transactivation/derepression of E2F-dependent proapoptotic genes. Cdk inhibitors prevent activation of the kinases and protect neurons from undergoing apoptosis.

for the normal development and survival of terminally differentiated neurons. Studies using the inhibitors of cdks and overexpression of Rb suggest that maintaining functional Rb in the cerebellum is important for its normal development and functioning. These studies therefore suggest that gene therapy using Rb should be considered for therapeutic intervention of diseases of the cerebellum, such as the ataxias and medulloblastomas, where Rb inactivation and cell cycle activation are closely associated.

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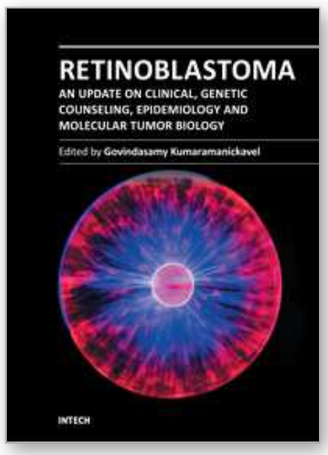

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Retinoblastoma is the first tumor suppressor gene discovered ever. The discovery opened a new avenue in the field of oncology leading to the identification of 35 tumor suppressor genes, till date in our genome. This book is an excellent compilation of both clinical and basic science information that meets the needs of a young clinician and a researcher at the same time. It also has abundant information on recent advances and cutting-edge knowledge in intracellular molecular cross-talking of retinoblastoma protein with various cellular viral-like proteins.

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